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EXHIBIT 4

Recombinant core particles of hepatitis B virus exposing fc antigenic determinants on their surface

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Insertion of foreign oligopeptide sequences (40-50 amino acids in length) into the Pro_{144} position of hepatitis B core antigen (HBcAg) leads to the formation of chimeric capsids in *Escherichia coli* cells. These capsids are morphologically and immunogically similar to native HBcAg, but expose the inserted oligopeptides on their outer surface and exhibit antigenic and immunogenic characteristics of the latter. As a source of model antigenic determinants, the appropriate DNA copies excised from cloned viral genes such as the pre-S region of hepatitis B virus, the transmembrane protein gp41 of human immunodeficiency virus 1 and the envelope protein gp51 of bovine leukemia virus have been used. The localization of the inserted antigenic determinants on the surface of chimeric capsids does not depend on the presence or absence of the arginine-rich, 39 amino acid-long C terminus of HBcAg.

Hepatitis B core antigen; PreS region; Human immunodeficiency virus-1 transmembrane protein gp41; Bovine leukemia virus envelope protein gp51; Antigenic determinant

1. INTRODUCTION

Expression of the core antigen (HBcAg) gene of hepatitis B virus (HBV) in *Escherichia coli* leads to the highly efficient synthesis of capsids, 25-27 nm in diameter, that are morphologically and immunologically indistinguishable from viral core particles [1-4]. Recently, the usefulness of recombinant HBcAg as a carrier for foreign oligopeptide sequences has been suggested [5-8]. Furthermore, we have constructed special vectors, so-called 'exposing vectors' [5,7] for insertion of the synthetic or natural DNA fragments coding for functionally important oligopeptides (e.g. antigenic determinants, peptide hormones, etc.) into preselected points of the HBcAg gene. The peptide sequences inserted at these points should (i) expose themselves on the outer surface of the capsid; (ii) preserve their native conformation and thus their antigenic and immunogenic properties; and (iii) preserve the capsid-forming ability of chimeras.

The most prospective in terms of preserving the capsid-forming ability seems to be the position 144 (Pro_{144}) [5] that lies close to the processing point (Thr_{147} or Val_{149}) of C polypeptide [9]. Such processing involves splitting off of the 34-36 amino-acid-long (subtype *ayw*) arginine-rich C terminus of HBcAg and results in the formation of hepatitis B-e antigen (HBeAg), not found to form capsid structures in vivo [9]. However, expression of a truncated HBeAg-like gene in *E. coli* leads to the efficient synthesis of self-assembled particles that are morphologically very similar to HBcAg capsids [5,7]. Moreover, direct analysis of HBcAg capsids by high resolution ^{15}N-NMR spectroscopy and monoclonal antibody mapping indicates the high spatial mobility and exterior localization of the C-terminal arginine-rich part of the molecule [10].

Here we report first immunological evidence for outer exposure of foreign antigenic determinants inserted into position Pro_{144} of the HBcAg gene. Different viral gene fragments coding for well-characterized antigenic determinants from such proteins as the pre-S region of HBV, transmembrane protein gp41 of human immunodeficiency virus 1 (HIV-1), envelope protein gp51 of bovine leukemia virus (BLV), were chosen as model objects. The appropriate DNA fragments were excised from cloned viral genomes and inserted into the polylinker sequence positioned between Pro_{144} and Glu_{145} in the exposing vector.

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2. EXPERIMENTAL

Recombinant plasmids were constructed by a combination of standard techniques on the basis of exposing vector pHBc1315, which contains the HBcAg gene controlled by the *trp* promoter and has (i) an optimized translation initiation region; and (ii) a polylinker sequence (*Eco*RV-*Cla*I-*Pvu*I) coding for 12 amino acids (KRSISKRSISIS) and inserted at the *Msp*I site which overlaps the Pro_{144} codon [7]. The following genomes served as sources of viral sequences: HBV, subtype *ayw* [11,12], BLV [13], HIV-1 [14]. The construction strategy is shown in fig.1. Cultures of *E. coli* strain K802 harboring the appropriate plasmids were grown by shaking overnight at 37°C to an OD_{650} of 4.0 in Casamino acid medium. The bacteria were lysed by lysozyme treatment and capsids were purified by Sepharose CL4B chromatography.

The molecular masses of chimeric polypeptides were determined by standard immunoblotting procedure after Laemmli's SDS-PAGE separation. Human polyclonal or murine monoclonal anti-HBc antibodies [10], monoclonal anti-preS1 MA18/7 [15], anti-preS2 mAb E and mAb F [16], anti-gp41 mAb 3D6 [17], anti-gp51 MAK14 [18] were used. The antigenicity of inserted epitopes was measured by two different ELISA variants when purified chimeric capsids or human or mouse anti-HBc were absorbed on the solid phase.

For electron microscopy, the samples were negatively stained with 1% aqueous uranyl acetate or 2% phosphotungstic acid and examined in a JEM 100B electron microscope at an accelerating voltage of 80 kV and a screen magnification of 100 000 ×. Immunogold electron microscopy was carried out as described [19].

DNA sequences were determined by the Sanger technique using synthetic oligonucleotide primers.

3. RESULTS AND DISCUSSION

Fig.1 shows the constructed chimeras with foreign oligopeptides inserted at Pro_{144} of HBcAg. Two variants of protein design are examined: (i) conserving the arginine-rich C terminus of the carrier molecule; and (ii) removing it. In all cases the inserted sequences are similar in size, approximately 40–50 amino acids, but vary strongly in their primary and also in predicted secondary structure (not shown).

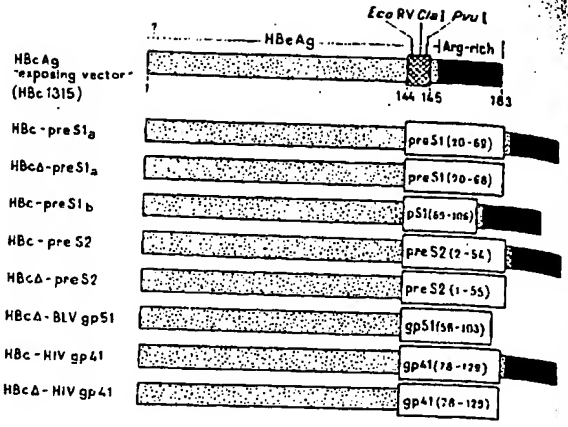

Fig.1. The principal scheme for construction of chimeric structures using HBcAg as a carrier.

The yield of chimeric polypeptides synthesized in *E. coli* cells resembles that of wild-type HBcAg and constitutes 10–25% of the total cellular protein. The chimeric products have the expected length for the given constructions (not shown) and appear exclusively in capsid-like particles already undergoing self-assembly in host cells (fig.2). Chimeric capsids are similar in their shape and diameter (about 25 nm) to initial HBcAg and differ from it only very slightly. They possess normal HBc-antigenicity, appearing as a complete coincidence of immunoprecipitation lines in the Ouchterlony double-diffusion test against human anti-HBc antibodies (not shown).

Selection of potential antigenic inserts was made on the basis of their practical importance as a possible source for diagnostics and vaccines. The latter is especially intriguing because our carrier particles harboring HBc- and HBe-antigenicity are potentially pro-

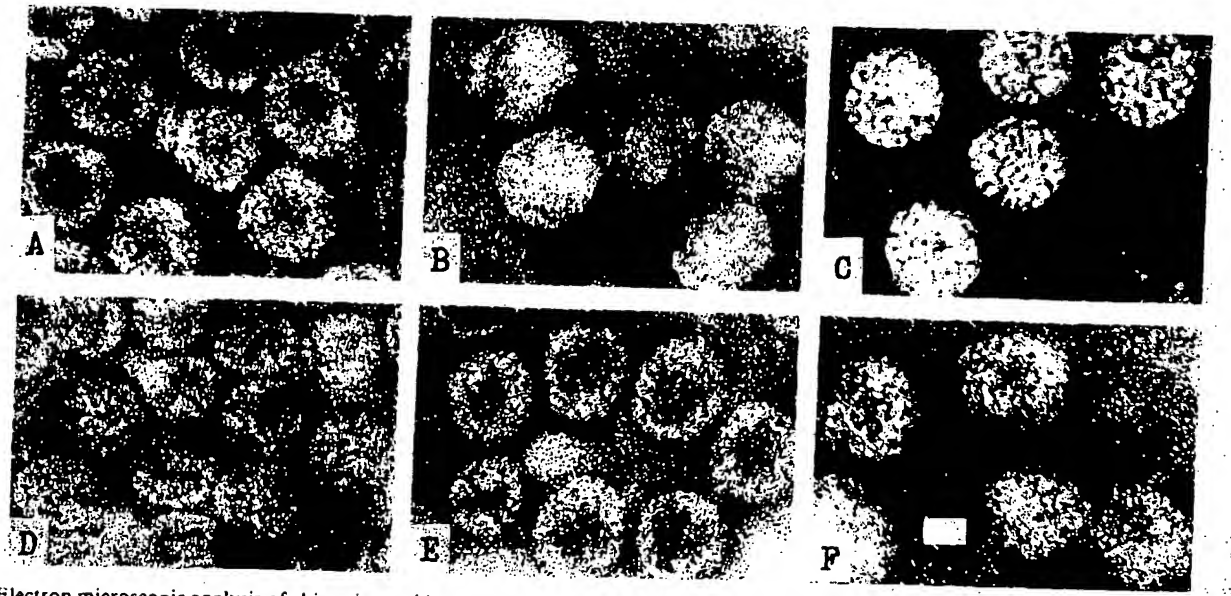

Fig.2. Electron microscopic analysis of chimeric capsids on the basis of HBcAg. (A) control (wild-type HBcAg); (B) HBcΔ-preS1a; (C) HBc-preS2; (D) HBcΔ-preS2; (E) HBcΔ-BLVgp51; (F) HBc-HIVgp41. Magnification 500 000 ×. White bar 10 nm.

Table 1

Antigenic properties of chimeric HBcAg capsids in ELISA assay

Capsids absorbed on the solid phase	P/N[a] ratio with subsequent monoclonal antibodies				
	Anti-HBc	Anti preS1	Anti-preS2	Anti-gp41	Anti-gp51
HBcAg	21	1	2	1	1
HBc-preS1$_a$	20	28	1	ND[b]	ND
HBcΔ-preS1$_a$	20	25	1	1	1
HBc-preS1$_b$	19	1	1	ND	ND
HBc-preS2	20	1	21	1	1
HBcΔ-preS2	20	1	22	ND	ND
HBc-HIVgp41	18	ND	ND	23	ND
HBcΔ-BLVgp51	18	ND	ND	ND	45

[a] Absorbance A_{492} ratio of specimens measured (P) and negative control (N)
[b] ND, not determined

HBc, which selectively recognizes only conformational HBc-epitopes. Nevertheless, antigenic properties of chimeras differed only slightly from those listed in table 1.

Further evidence for the surface localization of inserted oligopeptides was obtained by using immunogold electron microscopy (fig.3). Anti-species antibodies, labelled with colloidal gold, formed a typical halo around capsids that were able to bind the appropriate anti-epitope antibodies.

Chimeric capsids possess not only antigenic but also immunogenic properties of inserted sequences. After immunization of rabbits, both anti-HBc and anti-epitope antibodies have been found (fig.4). The level of anti-HBc immune responses in all cases corresponds to that obtained in a control after immunization with HBcAg. However, the titers of anti-epitope antibodies

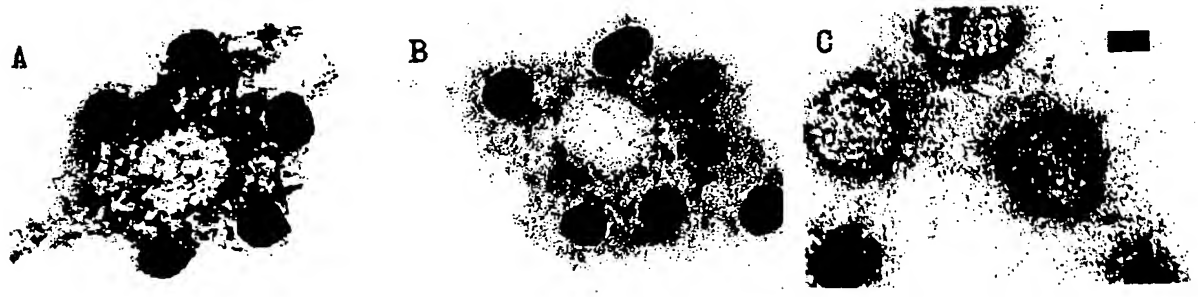

Fig.3. Immunogold electron microscopy of HBcAg chimeras. (A) HBcΔ-preS1$_a$ + anti-preS1 MA18/7; (B) HBc-preS2 + anti-preS2 mAb E; (C) HBcAg + MA18/7 (control). Magnification 500000 ×. Bar 10 nm.

fective in immunization against hepatitis B [20]. Moreover, HBcAg can serve as a T-cell and B-cell immunogen and shows high efficiency of T-cell priming [21]. Our choice of antigens was influenced also by the availability of well-characterized monoclonal antibodies directed against sequential epitopes of preS, gp51 and gp41 proteins. These antibodies were used to localize the inserted oligopeptides on the surface of HBcAg capsids.

When examined with the immunoblot technique, chimeric polypeptides displayed not only standard HBc-antigenicity (with monoclonal anti-HBc targeted sequential epitopes), but also the expected antigenicity of oligopeptides inserted with the appropriate monoclonal antibodies (see section 2).

Chimeric capsids, when absorbed on solid phase in ELISA assay, are recognized equally well not only by anti-HBc (monoclonal or polyclonal, conformational or sequential) but also by corresponding antibodies directed against the inserted epitopes (anti-epitope antibodies) (table 1).

To more reliably confirm the external positioning of inserted epitopes on the surface of native chimeric capsids, we have used an alternative ELISA technique, which restricted the absorption of non-assembled protein. The solid phase was coated in this case with anti-

are markedly lower. Moreover, the inserted oligopeptides show different immunogenicity despite the equal HBc-immunogenicity of chimeric capsids. Although chimeras present multiple copies of inserted oligopeptides on their surface, the appropriate immune responses in rabbits are lower than expected. Experiments to clarify this discrepancy are in progress.

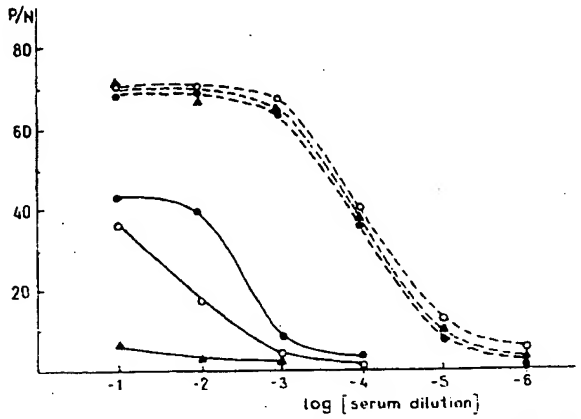

Fig.4. Immunogenic properties of HBcAg chimeras. HBc-preS proteins are shown as an example: HBc-immunogenicity as broken lines, preS-immunogenicity as solid lines. The following antigens were used for immunization: HBc-preS1$_b$ (○), HBcΔ-preS2 (●), HBcAg (▲).

In conclusion, HBcAg may serve as a carrier for foreign oligopeptide sequences of medium size, at least 40–50 amino acids long. These oligopeptides can be inserted into HBcAg before the arginine-rich C terminus or replace the latter without (i) influence on carrier self-assembly; or (ii) distortion of native conformation of inserted oligopeptides. This new approach could be used to create prospective immunodiagnostic reagents and polyfunctional vaccines against diseases of different etiology.

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